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In Re the Application of:

BERRY et al.

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For: "PROCESS FOR PRODUCTION OF N-GLUCOSAMINE"

Group Art Unit: 1652

Examiner: Fronda, C.

DECLARATION OF MING-DE DENG
(Under 37 CFR 1.132)

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being transmitted via facsimile to the United States Patent and Trademark Office on the date shown below.

CONSTANCE ROBNETT
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Signature: Constance Robnett

Date: 19 February 2002

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Ming-De Deng, declare as follows:

1. I am an investigator employed by the Assignee of the above-identified application, I have worked with the inventors of the application with regard to the subject matter claimed therein, and I am familiar with the application.

2. This Declaration is being submitted in conjunction with a Supplemental Amendment and Response to a final Office Action mailed July 17, 2001.

3. The following paragraphs (4) and (5), Tables 1 and 2, and Figures 1 and 2 are presented in support of the claims of the present invention, which are directed to method to produce glucosamine by fermentation. Specifically, the present discussion and data are submitted in support of the phrase "glucosamine-6-phosphate synthase" as used in the claims, in order to demonstrate that glucosamine-6-phosphate synthases from a variety of sources can be used successfully to practice the claimed method.

Specifically, to demonstrate the breadth of the claimed invention, the present inventors and the Assignee of the present application have functionally expressed different glucosamine-6-phosphate synthase genes in *E.coli* and have demonstrated a glucosamine production level in the

modified microorganisms that is higher than in wild type *E. coli*. The genes used in the following experiment were selected from representative species of diverse groups of organisms, such as gram-positive bacterium (*B. subtilis*), and yeast (*C. albicans* and *S. cerevisiae*). The overexpression of an *E. coli* glucosamine-6-phosphate synthase according to the claimed method has previously been demonstrated in the above-identified application. The glucosamine-6-phosphate synthase sequence homology shared by these organisms and *E. coli* is low (all in the range of about 40%), yet overexpression of each of the glucosamine-6-phosphate genes in an *E. coli* host cell resulted in a significant increase in glucosamine production by the host. Therefore, the data described in detail below provides strong evidence that any glucosamine-6-phosphate synthase gene can be used for glucosamine production in any host using the claimed method of the present invention.

4. *Sequence Homology Among Glucosamine-6-Phosphate Synthase Enzymes from Different Organisms*

By the end of 1997, and therefore at the time of the present invention, genes encoding glucosamine-6-phosphate synthase, also known as glucosamine:fructose-6-phosphate amidotransferase, were identified in many different organisms. The bacterial genes are named *glmS* and the eukaryotic homologues are called GFA, GFAT or GFPT. Organisms in which a glucosamine-6-phosphate synthase gene was identified at the time of the invention include Gram negative bacteria, Gram positive bacteria, yeast, nematode, mouse and human.

Representative glucosamine-6-phosphate synthase amino acid sequences were selected from representative organisms and aligned using the *E. coli* GlmS sequence as a reference. The sequence identities of the organisms are shown in Table 1. Also listed are the number of amino acid residues and deduced molecular weight of the selected enzymes. As shown in Table 1, the homology between *E. coli* GlmS and other sequences is in the range from 41.1% to 72.5%. The GFAT sequences in two yeast strains, *Candida albicans* and *Saccharomyces cerevisiae*, share 72.3% identical residues. The human GFAT sequence shows a homology of 42.1%, 57.5% and 99% to *E. coli*, *S. cerevisiae* and mouse sequences, respectively. The *E. coli glmS* nucleotide sequence shares 45 to 66.4% homology with other sequenced glucosamine-6-phosphate synthase genes listed in Table 1.

Despite differences in sequence, all characterized glucosamine-6-phosphate synthases share many similar features. For example, their $K_m$ to glutamine, $K_m$ to fructose-6-phosphate and optimal pH are very similar. At the amino acid sequence level, seven residues were shown to be important in catalysis: cysteine-1, asparate-29, histidine-86, histidine-97, asparate-123, cysteine-300 and lysine-603 (*E. coli* GlmS residue numbering). These residues are well conserved in sequences from different organisms. The initiator methionine in glucosamine-6-phosphate synthases is removed enzymatically after translation since the residue is not present in the mature proteins purified from *E. coli, T. thermophilus* and rat. The consensus sequence for the N-terminus is Cysteine-Glycine-Isoleucine. In fact, a cysteine is present at the N-terminal extremity of the mature form of all class-II GATase proteins (class of enzymes to which glucosamine-6-phosphate synthases belong). The cysteine residue has been shown to be important for the catalytic mechanism. The prosite PS00443 describes the specific amino acid residue pattern at the N-terminal of mature class-II GATase proteins.

Therefore, even with low sequence homology, there are conserved structural and biochemical features among the glucosamine-6-phosphate synthases that result in similar function of the enzymes.

5. *Expression of Different Glucosamine Synthase Genes for Glucosamine Production in E. coli*

Glucosamine-6-phosphatesynthase genes from bacteria(*glmS*) and yeast (GFA) were cloned and expressed in *E. coli* to demonstrate their utility in glucosamine production as claimed in the present application. The *glmS* and GFA coding sequences were amplified from *Bacillus subtilis, Saccharomyces cerevisiae* and *Candida albicans* by PCR and placed under the T7 promoter control in the expression vectors pET24d(+) or pET23b(+). The constructs were transformed into the *E. coli* strain 7107-17 (DE3) and maintained as free replicating plasmids. Cell cultures of different strains were induced with IPTG and evaluated for protein expression, glucosamine synthase activity and glucosamine production upon IPTG induction.

The *B. subtilis glmS* gene contains an open reading frame of 1803 bp and encodes a protein of about 65 kDa (599 residues, excluding the initiator methionine which is usually removed in the

cells). The *glmS* gene was amplified by PCR from the strains ATCC 23856 and ATCC 23857. PCR products of expected size were ligated into pET24d(+) (Novagen Inc, Wisconsin). The recombinant plasmids were confirmed by restriction analysis and transformed into 7101-17 (DE3), generating *E. coli* strains 7107-24 (*glmS* gene from *B. subtilis* ATCC23856) and 7107-25 (*glmS* gene from *B. subtilis* ATCC23857). As a control, the empty vector pET24d was also transformed into 7101-17(DE3), generating the strain 7107-22.

The *S. cerevisiae* GFA1 open reading frame has 2154 bp and encodes a peptide of 716 residues (excluding the initiator methionine). The protein size predicted from the sequence is about 80 kDa. There are no introns in the GFA1 gene sequence. Therefore, the gene was amplified from genomic DNA prepared from the strain *S. cerevisiae* S288C. The PCR product of about 2.2 kb was cloned into pCR-Script Amp SK(+). Recombinant plasmids were confirmed by restriction enzyme digestions. The *S. cerevisiae* GFA1 fragment was isolated by digestion with *Eco*R I and *Bsa* I and ligated into the *Eco*R I and *Nco* I sites of pET24d(+). The recombinant plasmid was confirmed by restriction analysis and transformed into 7101-17(DE3), generating the *E. coli* strain 7107-101.

The *C. albicans* GFA1 gene is free of introns and its 2142-bp open reading frame encodes a peptide of about 80 kDa (712 residues, excluding the initiator methionine). The GFA1 coding sequence was amplified from the strain ATCC10261 by PCR. The PCR product was cloned into the vector pMOSBlue (Amersham Pharmacia Biotech, New Jersey) and recombinant plasmids were confirmed by restriction enzyme digestion. The *Bsa* I-*Xho* I fragment was isolated and ligated into pET24d(+) prepared by digestion with *Nco* I and *Xho* I. The resulting plasmid was transformed into the host 7101-17 (DE3), generating the *E. coli* strain 7107-23.

The *C. albicans* GFA1 gene was also cloned into the expression vector pET23b (Novagen Inc). Unlike pET24d, this vector does not contain a *lacI* repressor gene and it does not have a *lac* operator sequence downstream from the T7 promoter. The use of this vector often results in a higher recombinant protein expression. The recombinant plasmid was confirmed by restriction analysis and transformed into the expression host 7101-17 (DE3), generating *E. coli* strains 7107-58 and 7107-59. As a control, the empty vector pET23b was also transformed into 7101-17(DE3), generating the strain 7107-57.

Strains transformed with pET vectors containing different *glmS* and GFA 1 genes described above were grown in LB medium and induced with 1 mM IPTG to demonstrate GlmS and GFA1 protein expression. As a negative control, cells with the empty pET24d vector were also grown and analyzed. For comparison, *E. coli* cells with the wild-type *E. coli glmS* gene and mutant *glmS*54* gene driven by the T7 promoter and integrated in the chromosome at the *lacZ* site were also grown and analyzed.

To investigate the expression level of the enzymes, SDS-PAGE was carried out by following standard methods. When the *T7-E. coli glmS* expression cassette was carried in pET plasmids or integrated in the chromosome, the GlmS protein was expressed at very high levels (Fig. 1, lane 2 and 3). Cells hosting the plasmids pET24d/*T7-B. subtilis glmS* over-expressed a protein of about 65 kDa, the expected size of the GlmS protein (lane 4 and 5). The expression level was comparable to the cells expressing the *E. coli glmS* gene contained in pET plasmids. Cells hosting the *S. cerevisiae* GFA gene showed an overexpressed protein band of the expected size for the yeast GFA1 protein (80 kDa, lane 6). In the strain 7107-23 containing the T7-*C. albicans* GFA1 expression cassette (lane 7), the synthesis of the 80-kDa protein band was not apparent when compared to the strain with the empty vector (lane 8). However, the GFA1 band was overexpressed in the strains 7107-58 and 7107-59 containing the *C. albicans* GFA1 gene carried by the vector pET23b (Fig. 2). The expression level was higher than in the strain 7107-23 with the pET24d-based vector. The use of alternative codons for Leu 29 and Ala 655 did not affect *C. albicans* GFA1 protein expression in *E. coli*. Expression levels of the yeast GFA genes in *E. coli* were low as compared to bacterial *glmS* genes. This is commonly observed when attempting to express eukaryotic genes in *E. coli* hosts. However, as discussed below, cultures expressing the yeast GFA genes still produced significantly increased amounts of glucosamine as compared to the controls.

For measurement of enzyme activity and glucosamine production, different strains were grown in M9A medium. Data from representative experiments are shown in Table 2. The enzyme activity was readily detectable in *E. coli* cells expressing the *B. subtilis glmS* genes. The activity level was comparable to the cells with a construct containing the *E. coli glmS* and *E. coli glmS*54* mutant

gene. A trace amount of enzyme activity could be detected in cells hosting the yeast GFA 1 genes, and this was correlated with the lower protein expression levels as discussed above.

Only a very low level of glucosamine was produced and secreted into the culture medium of 7101-17 (DE3) cells transformed with an empty vector pET24d (Table 1). Expression of a bacterial *glmS* gene (*E. coli glmS* or *B. subtilis glmS*) resulted in greater than 50-fold increase in glucosamine production. A several-fold increase in glucosamine level was also observed in the cultures expressing yeast GFA1 genes, demonstrating that even at a lower level of enzyme overexpression and activity as compared to the bacterial genes, *significant glucosamine production is achieved.* As compared to pET24d, the use of pET23b led to a higher level of *C. albicans* GFA1 protein and a higher level of glucosamine production. As observed in enzyme activity assays, integration of the *T7-E. coli glmS* expression cassette in the chromosome appeared to be beneficial, as a higher glucosamine level was produced in the strain 2123-12 than in 7107-214. The *E. coli* strain with *E. coli glmS*54* integrated in the chromosome was the highest for glucosamine production when compared to other tested strains.

In summary, these experiments demonstrate that, using the guidance provided in the present application, the method claimed in the above-identified application can be predictably and successfully practiced using glucosamine-6-phosphate synthase from a variety of organism sources.

6. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

Date: Feb 15, 2002 By: _____

Ming-De Deng

## Table 1. Amino Acid Sequence Comparison of Glucosamine Synthases from Different Organisms*

*: Sequences were published before January, 1998

**: Number of amino acid residues does not include the initiator methionine, which is removed enzymatically after translation.

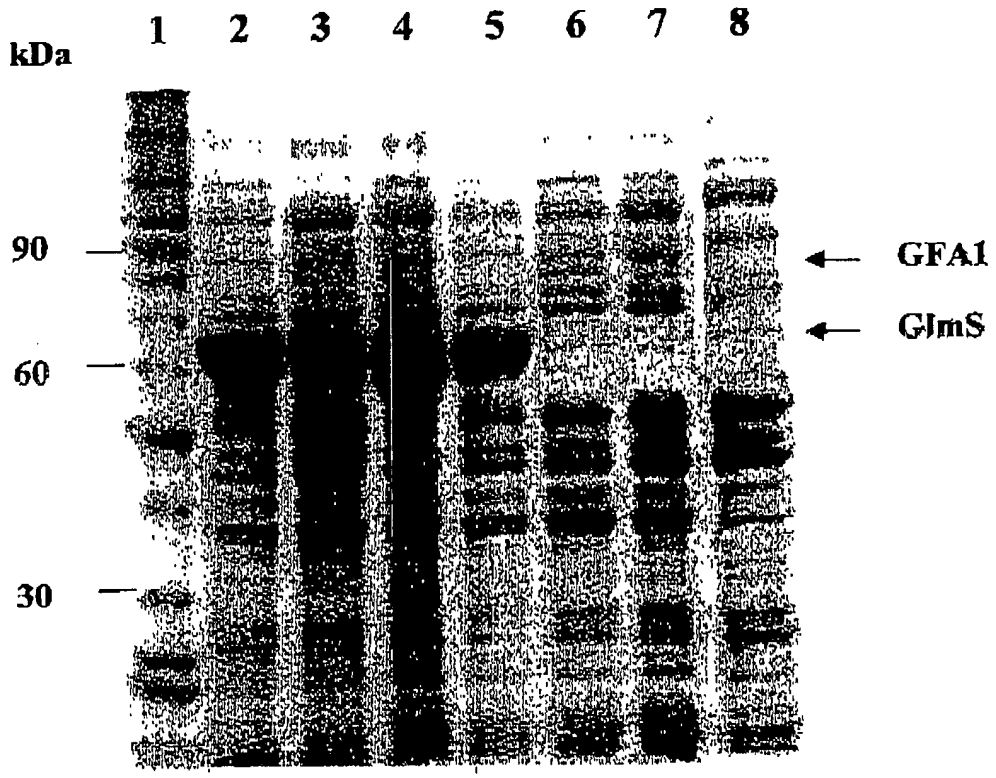
***: Percentage of identical residue was determined by the Method of J Hein, Lasergene software

| | Sequence Name | Number of Residues** | Molecular Weight (kDa) | Identical Residues (%)*** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1 | *E. coli* GlmS | 608 | 66.8 | | 72.5 | 47.7 | 49.2 | 44.0 | 41.1 | 46.6 | 42.1 | 42.1 | 41.1 | 41.7 | 42.1 |
| 2 | *H. influenzae* GlmS | 609 | 66.7 | | | 46.8 | 49.1 | 42.8 | 40.0 | 45.4 | 42.0 | 41.3 | 40.7 | 43.0 | 43.3 |
| 3 | *R. leguminosarum* GlmS | 607 | 65.7 | | | | 48.3 | 44.3 | 41.2 | 44.2 | 38.2 | 38.7 | 37.7 | 39.7 | 39.5 |
| 4 | *T. thermophilus* GlmS | 603 | 66.4 | | | | | 50.4 | 43.9 | 47.1 | 39.5 | 38.6 | 39.4 | 40.0 | 39.8 |
| 5 | *M. leprae* GlmS | 624 | 67.4 | | | | | | 40.1 | 41.6 | 37.9 | 37.8 | 38.2 | 38.1 | 37.8 |
| 6 | *B. subtilis* GlmS | 599 | 65.2 | | | | | | | 40.3 | 36.1 | 35.6 | 34.1 | 35.6 | 35.9 |
| 7 | *Synechocystis sp* GlmS | 630 | 69.5 | | | | | | | | 38.9 | 37.7 | 37.5 | 38.6 | 38.6 |
| 8 | *C. albicans* GFA1 | 712 | 79.1 | | | | | | | | | 72.3 | 52.7 | 56.4 | 56.6 |
| 9 | *S. cerevisiae* GFA1 | 716 | 79.9 | | | | | | | | | | 53.6 | 57.3 | 57.5 |
| 10 | *C. elegans* GFAT | 709 | 79.2 | | | | | | | | | | | 61.3 | 61.0 |
| 11 | Mouse GFAT1 | 680 | 76.6 | | | | | | | | | | | | 99.0 |
| 12 | Human GFAT1 | 680 | 76.6 | | | | | | | | | | | | |

## Table 2. Glucosamine synthase activity and glucosamine production in *E. coli* strains expressing different *glmS* and GFA homologues

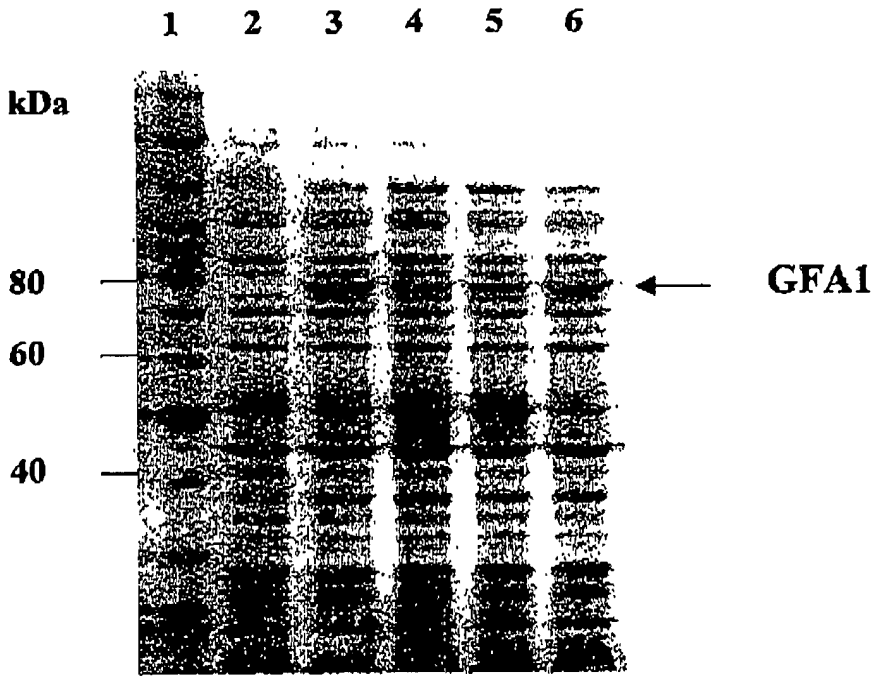
| Strain Number | Strain description | Enzyme activity ($nmol.min^{-1}.mg^{-1}$) | Glucosamine ($mg.l^{-1}$) |
|---|---|---|---|
| 7107-22 | pET24d | trace | 5 |
| 7107-24 | pET24d /*T7-B. subtilis glmS* 23856 | 637 | 128 |
| 7107-101 | pET24d/*T7-S. cerevisea* GFA1 | trace | 47 |
| 7107-23 | pET24d/*T7-C. albicans* GFA1 | trace | 23 |
| 7107-58 | pET23b/*T7-C. albicans* GFA1 | trace | 54 |
| 7107-60 | pET23b/*T7-C. albicans* GFA1-M | trace | 58 |
| 7107-214 | pET24d/*T7-E. coli glmS* | 297 | 37 |
| 2123-12 | *lacZ::T7-E. coli glmS* | 613 | 75 |
| 2123-54 | *lacZ::T7-E. coli glmS*54* | 803 | 2,029 |

Notes: 1) Host cell: *E. coli* 7101-17 (DE3). Genotype: *nag, manXYZ* DE3.
2) Cell culture: 30°C for 26 hrs in shake flasks containing M9A medium supplemented with 7.5 g $(NH_4)_2SO_4$ per liter and 40 g glucose per liter.
3) *C. albicans* GFA1 (M): Leu 29 and Ala 655 codons changed from TTA and GCT to CTA and GCC, respectively.




1: Size Standard
2: 7107-214 (pET24d/*T7-E. coli glmS*)
3: 2123-12 (*lacZ::T7-E. coli glmS*)
4: 7107-24 (pET24d/*T7-B. subtilis glmS* 23856)
5: 7107-25 (pET24d/*T7-B. subtilis glmS* 23857)
6: 7107-101 (pET24d/*T7-S. cerevisiae* GFA1)
7: 7107-23 (pET24d/*T7-C. albicans* GFA1)
8: 7107-22 (pET24d, control)

**FIG. 1**




1: protein size markers
2: 7107-57(pET23b)
3: 7107-58 (pET23b/T7-*C. albicans* GFA1)
4: 7107-59 (pET23b/T7-*C. albicans* GFA1)
5: 7107-60 (pET23b/T7-*C. albicans* GFA1-M)
6: 7107-61 (pET23b/T7-*C. albicans* GFA1-M)

**FIG. 2**